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Distinctive Cytoskeletal Organization in Erythrocytes of the Cold-Seep Vesicomyid Clam, *Calyptogena kilmeri*

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Abstract. Erythrocytes have long served as model cells, useful for analyzing cytoskeletal structure and function. In non-mammalian vertebrates, erythrocytes are typically highly flattened, nucleated ellipsoids in which a marginal band (MB) of microtubules interacts with the membrane skeleton (MS) to generate and maintain cell shape. Though relatively rare, erythrocytes also occur in representatives of many invertebrate phyla, including the arcid and vesicomyid molluscs, but the structure and function of these cells are not well understood. Previous work has shown arcid erythrocytes to be highly flattened ellipsoids containing the MB-MS cytoskeletal system, similar to vertebrates but with an additional interesting feature: a functional centriole-containing centrosome associated with each MB. In the present study we have examined, for the first time, erythrocyte morphology and cytoskeletal structure in a vesicomyid. Using *Calyptogena kilmeri*, the dominant invertebrate at many Pacific cold seeps, we have found that the erythrocytes are only slightly flattened and do not contain MBs. Rather, their cytoskeletons display a peripheral centriole-containing centrosome with radiating fibers, a distinctive type of organization not observed previously in mature erythrocytes from any species.

Introduction

Hemoglobin-bearing erythrocytes are found in all vertebrates and have long been utilized for studies of cytoskeletal organization and function. In adult mammals the erythrocytes are typically anucleate discoids, with a cytoskeletal system consisting principally of the membrane skeleton (MS). In adult non-mammalian vertebrates and mammalian embryos the mature erythrocytes are nucleated, flattened ellipsoids or discoids, and the cytoskeleton characteristically includes a marginal band (MB) of microtubules. The MB resists mechanically and osmotically induced shape deformation in normal mature cells, and abnormally pointed MBs generate abnormally pointed cells, presumably by MS deformation (Joseph-Silverstein and Cohen, 1984). Mechanical interaction between these two cytoskeletal components is thus believed to be important for morphogenesis and maintenance of cell shape in nucleated erythrocytes (Cohen, 1991; Winckler and Solomon, 1991).

Invertebrates with erythrocytes are relatively rare, but phylogenetically diverse; they include representatives of the annelids, brachipods, echinoderms, echiurans, molluscs, priapulids, and sipunculans. These invertebrate erythrocytes vary with respect to morphology and type of respiratory protein, and the MB-MS cytoskeletal system may or may not be present. The role of these cells is incompletely understood, with several workers suggesting that erythrocytes may be multifunctional in some species (*e.g.,* Terwilliger *et al.,* 1985). Comparative studies of cytoskeletal organization in invertebrate erythrocytes might, therefore, help to shed light on the functions of these cells.

Among the molluscs, erythrocytes appear in representatives of the arcid and vesicomyid bivalves (Terwilliger *et al.,* 1983; Cohen and Nemhauser, 1985; Nagel, 1985). Previous work on arcid species—including *Anadara transversa, A. ovalis,* and *Noetia ponderosa* from coastal

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waters of the eastern United States—has demonstrated that the MB-MS system is present and functional in maintenance of cell shape (Cohen and Nemhauser, 1980; Nemhauser *et al.,* 1983; Joseph-Silverstein and Cohen, 1984, 1985). Each MB also has an associated centrosome containing a pair of centrioles, an unusual feature not observed in mature erythrocytes of vertebrates (Cohen and Nemhauser, 1980; Cohen, 1991). This centrosome remains functional as a microtubule organizing center—as demonstrated by experimentally induced MB reassembly in the living cells (Nemhauser *et al.,* 1983)—and is presumed to be the same centriole that was involved in MB biogenesis during erythrocyte differentiation. Erythrocytes of the Australian species *A. trapezia* and several related Japanese bivalves have a virtually identical centrosome-containing cytoskeleton (Ochi, O., and Cohen, W. D., 1984, and Cohen, W. D., 1989, unpubl. obs.), indicating that this feature is a general characteristic of arcid erythrocytes.

In contrast with the arcids, cytoskeletal structure in vesicomyid erythrocytes has never been described. Of those vesicomyids with erythrocytes, *Calyptogena magnifica* is best known because it populates well-explored Pacific hydrothermal vent communities (Boss and Turner, 1980; Johnson *et al.,* 1988b; Fisher *et al.,* 1988) and because its erythroid hemoglobin has been studied (Terwilliger *et al.,* 1983). Most (perhaps all) other *Calyptogena* species with erythrocytes inhabit "cold seeps," sites that have high levels of hydrogen sulfide, low levels of oxygen, and high hydrostatic pressures in common with hydrothermal vents (McHugh *et al.,* 1992; Barry *et al.,* 1997a; Vrijenhoek *et al.,* 1994). The principal difference between the two environments is that hydrothermal vents are characterized by fluctuating and frequently elevated temperatures (Johnson *et al.,* 1988a), whereas cold-seep temperatures are similar to those of surrounding waters and are relatively constant. Vesicomyid bivalves inhabiting cold seeps in the Monterey Canyon—including *Vesicomya gigas, V. steamsii, Calyptogena kilmeri,* and *C. pacifica*—experience a temperature range of only 4–6°C (Barry, J. P., pers. comm.). Of these species, *C. kilmeri* is the most abundant at several cold-seep sites routinely sampled by the remotely operated vehicles (ROVs) of the Monterey Bay Aquarium Research Institute.

Seeking to determine whether molluscan erythrocytes have a common cytoskeletal organization, we undertook to compare the structure of vesicomyid erythrocytes with that known for erythrocytes of the arcids. We report here the first examination of cell morphology and cytoskeletal structure in vesicomyid erythrocytes, those of *C. kilmeri.*

Materials and Methods

Experimental material

Clams (*C. kilmeri,* Fig. 1) were collected by the ROV *Ventana* from the Monterey Bay cold-seep locale known as "Clam Field" (Barry *et al.,* 1997b). They were maintained for one to several days in laboratory seawater tanks at 6°C, approximately the temperature of their native habitat. The animals were opened with a scalpel, and the bright red, flowing blood (hemolymph) was collected in a plastic tray and used immediately in experiments. Depending on the experiment, hemolymph was collected either at 22°C (room temperature) or at 6°C (coldroom).

Figure 1. *Calyptogena kilmeri.* Animals were collected by a remotely operated vehicle (ROV) and maintained in aquaria in a closed filtered seawater system at 6°C.

Erythrocyte cytoskeletons were prepared, in general, by lysis of cells with nonionic detergents under microtubule-stabilizing conditions. Immediately after collection (<30 s) the hemolymph was diluted about 1:20 into Brij lysis medium or Triton lysis medium. The Brij lysis medium contained 100 m*M* piperazine-N,N′-bis(ethanesulfonic acid) [PIPES buffer], 5 m*M* ethylene glycol-bis-(β-aminoethyl ether) n,n′-tetraacetic acid [EGTA], 1 m*M* $MgCl_2$, pH 6.8, plus 0.6% Brij-58. The Triton lysis medium was similar except that it contained 0.4% Triton X-100 instead of Brij-58. In some cases, 0.1% glutaraldehyde was included in the medium for additional rapid post-lysis stabilization. These media had been effective previously for preparing erythrocyte cytoskeletons from a wide range of vertebrates and invertebrates, including the archid genera *Noetia* and *Anadara* (Nemhauser *et al.,* 1983; Cohen and Nemhauser, 1985; Cohen, 1991).

Microscopy

Cells for morphological examination were either unfixed, or fixed immediately (<30 s) by dilution (about 1:20) of hemolymph into marine molluscan Ringer's solution (Cavanaugh, 1975) containing 0.1% glutaraldehyde. Information on cell shape was obtained by observing cells while they were stationary in the medium on a slide, as well as while they were tumbling in flow. Observations

and photomicrographs of the cells and their cytoskeletons were made with a Zeiss Axioscope equipped with an Olympus 35-mm camera system with focusing eyepiece, and phase contrast optics including a 100× Plan-Neofluar objective (NA 1.3).

For transmission electron microscopy of cytoskeletons, fresh hemolymph obtained at 6°C was diluted into Triton lysis medium containing 0.1% glutaraldehyde, incubated 1 h at about 22°C, stored 3 days at 0°C with glutaraldehyde added to 1%, and post-fixed 1 h in 1% OsO_4 buffered with 0.1 *M* KH_2PO_4-KOH at pH 6.8. After ethanol dehydration, the material was embedded in Polybed 812 (Polysciences, Inc.), thin sectioned with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-600 transmission electron microscope.

Results

Cell morphology

The erythrocytes were generally ellipsoidal, but irregular in size and contour (Fig. 2a). Observations made as they tumbled in flow under coverslips showed that the cells were somewhat flattened, but relatively thick (Fig. 2b, c). The shape of the *C. kilmeri* erythrocytes was the same whether examined in living cells or in cells fixed immediately upon collection of the hemolymph.

Cytoskeletal structure

In different experiments, erythrocyte cytoskeletons were prepared from hemolymph collected either at room temperature (~22°C) or at cold-seep temperature (cold-room at ~6°C); the lysis media were also maintained either at ~22°C or ~6°C. Examination by high resolution, phase contrast light microscopy revealed, in most cells, a peripheral pair of closely opposed phase-dense "dots" of uniform size (Fig. 3a–d; arrowheads). In highly flattened samples, this structure was clearly resolvable as a centriole-containing centrosome from which straight fibrous material radiated (Figure 3e-g). However, circumferential MBs of microtubules were not present.

The cytoskeletal structure observed was not induced by temperature during the experimental procedure. Peripheral pairs of centrioles with radiating fibers were observed in the cytoskeletons whether the hemolymph was collected at ~22°C or ~6°C, or whether lysis was achieved with media at either temperature. We did note, however, that cytoskeletons tended to collapse closer to the nucleus more frequently when the cells were collected and lysed at the lower temperature; thus, our stabilization media were not as effective at that temperature.

Although the paired, phase-dense "dots" were similar to those observed previously in phase contrast and subsequently identified as centrioles in *Noetia* and *Anadara* (Cohen and Nemhauser, 1980, 1985), transmission electron microscopy of thin sections was used to verify their identification in *Calyptogena*. The cytoskeletons were found to contain classic pairs of centrioles measuring about 0.22 × 0.32 μm (Fig. 4a–c), with typical 9-triplet ultrastructure (Fig. 4d), and microtubules observed frequently in their vicinity did not emanate directly from the centriolar triplets (Fig. 4a–c). In many cases a mass of electron-dense material was observed in association with one or both centrioles (*e.g.*, Fig. 4b, d, arrowheads).

Discussion

With their generally ellipsoidal and partially flattened irregular shape, the *C. kilmeri* erythrocytes differed considerably in morphology from those of arcids, which are much flatter and smoother in contour when first removed from the animal (Cohen and Nemhauser, 1985). In addition, the shape of the vesicomyid cells remained stable,

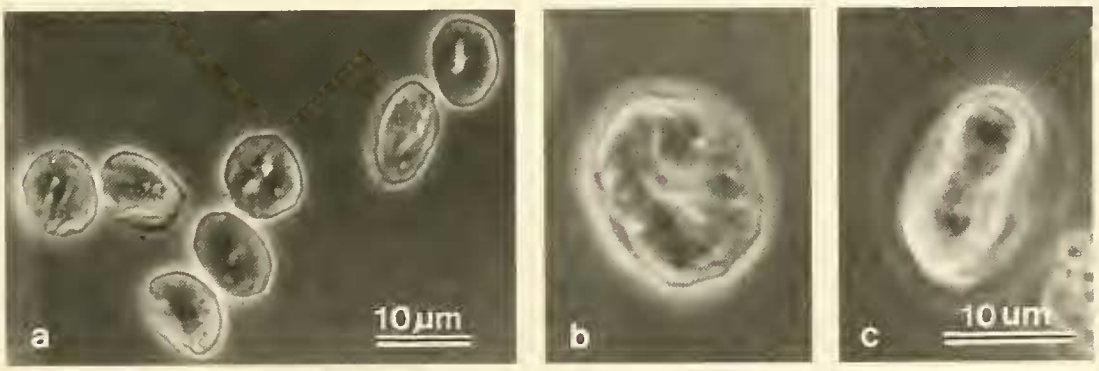

Figure 2. Fixed erythrocytes of *Calyptogena kilmeri*, observed by phase contrast microscopy. (a) Erythrocytes were generally ellipsoidal, but irregular in size and contour; (b, c) face and edge views, respectively, of the same cell, observed as it tumbled in flow. The erythrocytes are thus found to be somewhat flattened but relatively thick, and the shape of unfixed cells is similar (not shown).

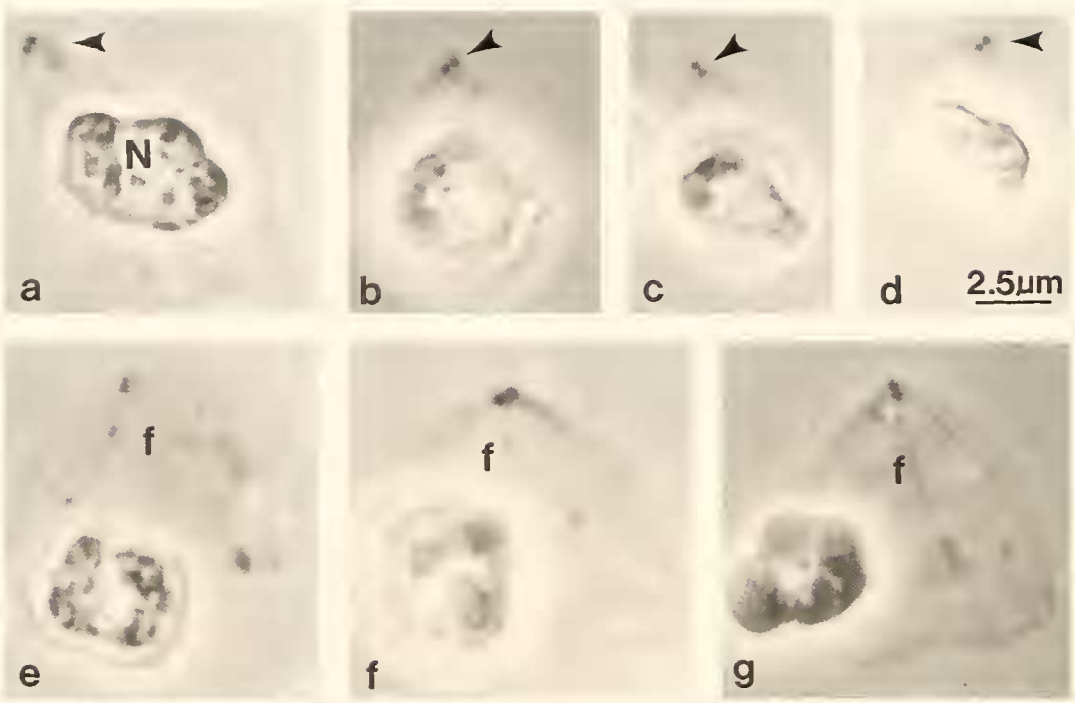

Figure 3. Cytoskeletons of *Calyptogena kilmeri* erythrocytes; phase contrast microscopy. (a–d) Examples of cytoskeletons in which centrioles are resolved as a pair of phase-dense "dots" (arrowheads); (e–g) cytoskeletons flattened under the coverslip to improve visualization of fibers (f) radiating from the centrosomal region. Marginal bands of microtubules are not present. Conditions: (a) and (e–g), Brij lysis medium, no glutaraldehyde; (b, c), Triton lysis medium + glutaraldehyde; (d), Brij lysis medium + glutaraldehyde.

whereas arcid erythrocytes, if left in their own hemolymph, undergo a spontaneous, reversible morphological transformation to lumpy spheroids within about 5 min (Sullivan, 1961; Dadacay *et al.*, 1996).

Cytoskeletal structure in these vesicomyid erythrocytes was distinctive. Fibers radiated from prominent centrosomes containing centriole pairs similar to those of arcid erythrocytes, but, in contrast to the arcid cells, the vesicomyid erythrocytes contained no MB. So far as we have been able to determine, this kind of organization has not been observed previously in erythrocytes of any other species. Our survey encompasses annelid, brachiopod, echinoderm, echiuran, molluscan, priapulid, and sipunculan representatives among the invertebrates (Table I), as

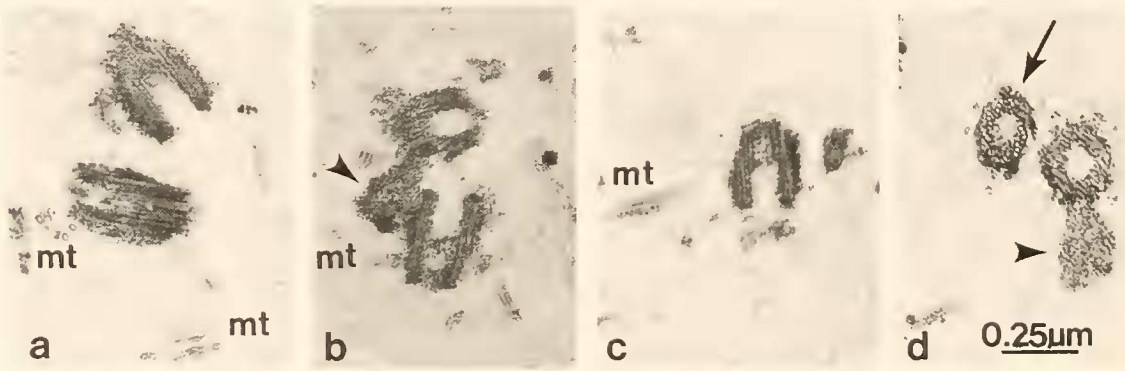

Figure 4. Centrioles in cytoskeletons as observed by transmission electron microscopy of thin sections. (a–c) Longitudinal and oblique views; (d) cross-sectional view showing 9-triplet cylindrical ultrastructure. Adjacent microtubules or microtubule bundles (mt) did not emanate from the centriolar triplets (a–c). Electron-dense material was sometimes observed close to the centrioles (b, d; arrowheads).

Table 1

Survey of cytoskeletal organization in invertebrate erythrocytes

Phylum,[1] species	RP[2]	MB[3]	PC[4]	Reference
Annelida				
Travisia japonica	Hb	+	+	Ochi, 1969; Ochi, unpubl. obs., 1980
Pista pacifica	Hb	−	−	Nemhauser, 1981; Terwilliger *et al.*, 1985
Glycera dibranchiata	Hb	−	−	Nemhauser, 1981; Pierce and Maugel, 1985
Brachiopoda				
Laqueus californicus	He	−	−	Nemhauser, 1981; Meglitsch, 1967
Echinodermata (Holothuroidea)				
Cucumaria miniata	Hb	+	?	Fontaine and Lambert, 1973
Molpadia arenicola	Hb	+	?	Clifford, 1969
Eupentacta quinquesemita[5]	Hb	+	+	Fontaine and Hall, 1981
Echiuroida				
Urechis caupo	Hb	−	−	Nemhauser, 1981; Terwilliger *et al.*, 1985
Mollusca (Arcidae & Vesicomyidae)				
Anadara transversa	Hb	+	+	Cohen and Nemhauser, 1985
A. ovalis	Hb	+	+	Nemhauser, 1981
A. lienosa	Hb	+	+	"
A. braziliana	Hb	+	+	"
A. trapezia	Hb	+	+	Cohen, W. D., unpubl. obs., 1988
Noetia ponderosa	Hb	+	+	Nemhauser *et al.*, 1983
Barbatia virescens	Hb	+	+	Cohen and Nemhauser, 1985; Ochi, O., and Cohen, W. D., unpubl. obs. 1984
Arca boucardi	Hb	+	+	"
Pseudogrammatodon dalli	Hb	+	+	"
Calyptogena kilmeri	Hb	−	+	this paper
Priapulida				
Priapulus caudatus	He	+	?	Mattison and Fange, 1973
Sipuncula				
Phascolopsis gouldii	He	+	?	Nemhauser *et al.*, 1980
Themiste dyscrita	He	−	−	Nemhauser, 1981[6]; Terwilliger *et al.*, 1985[7]

[1] Where erythrocytes are restricted to a class or family, this is indicated in parentheses.

[2] Abbreviations: RP = respiratory protein; Hb = hemoglobin; He = hemerythrin; MB = marginal band of microtubules; PC = peripheral centriole-containing centrosome.

[3] MB observed (+) or not observed (−).

[4] PC consistently observed (+) or not observed (−); ? = insufficient data.

[5] Cells with MBs and PC in this species are described as *immature* flattened ellipsoids.

[6] Only coelomic erythrocytes observed.

[7] Both coelomic and circulatory erythrocytes observed.

well as a wide range of vertebrates including fish, amphibians, reptiles, birds, newborn marsupials, and placental mammals (Cohen and Nemhauser, 1985; Cohen *et al.*, 1990). In many of the arcid species in Table 1, erythrocyte cytoskeletons bearing partial resemblance to those of *C. kilmeri* are found in natural subpopulations of singly pointed erythrocytes in which the centrosome occupies the pointed end. In these cytoskeletons, however, unlike those of *C. kilmeri*, the opposite end contains the curved portion of the incomplete MB. Cytoskeletons of arcid erythrocytes in the early stages of experimentally induced MB reassembly (Nemhauser *et al.*, 1983) also bear some resemblance to those observed in this study of *C. kilmeri* erythrocytes.

As observed by transmission electron microscopy, the centriole pairs were typical in dimensions and in frequent near right-angle orientation (*e.g.*, Fig. 4a, b). That microtubules in their vicinity were not continuous with centriolar triplets is consistent with cytoskeletal organization by centrosomes in other systems, based on pericentriolar distribution of gamma tubulin. However, as in the arcids, a distinctive surrounding region of pericentriolar material, such as is typical of mitotic centrosomes, was not observed in the *Calyptogena* erythrocyte cytoskeletons. Discrete electron-dense masses were frequently found in association with one or both centrioles (*e.g.*, Fig. 4b, d), but microtubules did not emanate from them. The question arises as to the relationship between these erythrocyte centrosomes and those of precursor cells. We assume that, both in arcid and vesicomyid erythrocytes, the peripheral centriole pairs are the same ones initially located more centrally at the erythroblast mitotic spindle poles, each

daughter cell having received one pair. This implies the existence of control mechanisms that reprogram or switch centrosome function from mitotic spindle organization to erythrocyte morphogenesis and shape maintenance.

Among the species in Table 1, in addition to *C. kilmeri*, there are five in which erythrocyte MBs are lacking. However, these species also lack the centrosomal organization found in *C. kilmeri* erythrocytes, verified by examination of cytoskeletons as in the present work (Nemhauser, 1981). The best studied of these cases are instructive. The hemoglobin-bearing erythrocytes of *Pista pacifica*, *Glycera dibranchiata*, and *Urechis caupo* are all spherical (Terwilliger *et al.*, 1985; Pierce and Maugel, 1985), and the coelomic hemerythrin-containing cells of *Themiste dyscrita* are described as varying from disks to spheres (Terwilliger *et al.*, 1985). Absence of the MB is thus correlated with absence or loss of morphological asymmetry. Conversely, MB presence is correlated with maintenance of marked erythrocyte flatness, as in the Arcidae (Cohen and Nemhauser, 1985). The unusual cytoskeletal organization of *C. kilmeri* erythrocytes might then be viewed as functioning to maintain an equally unusual intermediate morphological state, that of limited cell flattening.

The value of erythrocyte flattening to an organism can be at least twofold. First, reduction of diffusion distances is generally acknowledged to facilitate respiratory gas exchange, and second, flattening reduces the work required to maintain "blood" flow by reducing its viscosity (Fischer, 1978). The latter helps to explain why blood cells other than erythrocytes, including mammalian platelets, non-mammalian vertebrate thrombocytes, and invertebrate clotting cells such as *Limulus* amebocytes (Armstrong, 1985) are also highly flattened. All of these cell types also contain MBs. Conversely, for organisms with spherical erythrocytes, we might speculate that rapid respiratory gas exchange and the requirement for reduced viscosity are not as critical.

In interpreting the data, potential artifacts must be considered. We note that the habitat of *C. kilmeri* is characterized in part by relatively high pressures, with a depth range of ~0.5–5 km for cold-seep vesicomyids in general, and ~0.9 km at "Clam Field" (Orange, D. L., and Barry, J. P., pers. comm.). Since our findings were obtained from animals brought to sea level, it might be hypothesized that arcid-like MBs with associated centrosomes occur in vesicomyid erythrocytes under native conditions and become disorganized by the pressure reduction. We believe this to be quite unlikely; as shown in many studies (summarized in Dustin, 1978, 1984), it is *increased* hydrostatic pressure that can cause microtubule disorganization. Data relevant to this question might be obtainable by fixing material *in situ* at the time of specimen collection, but we have found that fixation of whole, unlysed bivalve erythrocytes (both arcid and vesicomyid) traps sufficient hemoglobin to obscure cytoskeletal elements in thin sections for transmission electron microscopy and to render immunofluorescence light microscopy ineffective. Indeed, blood must be withdrawn and the cells lysed, as in the present work, if the entire cytoskeleton is to be viewed. Such procedures would be problematic at the depths and under the challenging physical conditions characteristic of the cold seeps.

A second possible source of artifact would be a spontaneous change of erythrocyte shape occurring in the hemolymph shortly after collection, as observed previously in arcid erythrocytes (Cohen and Nemhauser, 1985; Dadacay *et al.*, 1996) and in coelomic erythrocytes of a sipunculan, *T. dyscrita* (Terwilliger *et al.*, 1985). In the present work, this is not a factor. Although, in the absence of prior data, precautions were taken by preparing cytoskeletons and fixed cells immediately upon obtaining the hemolymph, such shape alteration was not observed in the vesicomyid erythrocytes.

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